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Review

PAX3-FOXO1: Zooming in on an "undruggable" target

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ABSTRACT

Driver oncogenes are prime targets for therapy in tumors many of which, including leukemias and sarcomas, express recurrent fusion transcription factors. One specific example for such a cancer type is alveolar rhabdomyosarcoma, which is associated in the majority of cases with the fusion protein PAX3-FOXO1. Since fusion transcription factors are challenging targets for development of small molecule inhibitors, indirect inhibitory strategies for this type of oncogenes represent a more promising approach. One can envision strategies at different molecular levels including upstream modifiers and activators, epigenetic and transcriptional co-regulators, and downstream effector targets.

In this review, we will discuss the current knowledge regarding potential therapeutic targets that might contribute to indirect interference with PAX3-FOXO1 activity in alveolar rhabdomyosarcoma at the different molecular levels and extrapolate these findings to fusion transcription factors in general.

1. Introduction

Targeted therapies are generally believed to have the potential to revolutionize cancer therapy. Optimally, targeted drugs should affect cancer and spare normal cells in the body, thereby greatly enlarging the therapeutic window over currently used chemotherapy-based approaches. Prime targets for such drugs are altered (mutated or overexpressed) driver oncogenes present in a given tumor. Some of them are frequently expressed, such as EGFR mutations in non-small-cell-lung cancer (NSCLC) or mutant BRAF in melanoma, while other potentially actionable targets are expressed in a minority of patients or even in single individuals only. Analysis of the mutational landscape by next generation sequencing (NGS), as already implemented in the clinics for some entities, allows identification of such targets and helps to tailor patient specific therapies with targeted drugs [1]. However, in some tumor types, actionable targets are not (or only in rare cases) available and therefore this strategy is less helpful. Examples are several pediatric malignancies such as leukemia and sarcoma, which are associated with specific chromosomal translocations, generating fusion oncogenes. Typically, these are characterized by a simple, near-diploid karyotype and a very low mutational burden. In a surprisingly large number of cases, the translocations are the only cancer-associated aberrations identifiable, even by whole genome sequencing [2]. Moreover, clonal analyses revealed that other mutations, if present, are secondary to the translocation event and might therefore be expressed only in subsets of tumor cells [3]. Taken together, this demonstrates that the fusion proteins act as main drivers of tumorigenesis. Importantly, genetic loss-of-function studies in different cancers revealed that tumor cells are addicted to the activity of the fusion proteins and undergo cell death when they are depleted [4–6]. These results suggest that they are among the most promising targets for successful therapy.

Strikingly, only a minority of the known fusion proteins contain a druggable domain, such as kinase-domains present in BCR-ABL in chronic myelogenous leukemia (CML), or in ALK and NTRK translocations both found in different carcinoma, haematologic tumors, and sarcoma [7,8]. The great clinical success in treatment of CML patients with BCR-ABL inhibitors, but also the promising preliminary clinical experience with ALK and NTRK inhibitors [8–13], underscores the suitability and importance of fusion oncogenes as therapeutic targets in general. However, the great majority of fusion proteins act as transcription factors (TF) and are composed of domains from two TFs, transcriptional co-regulators or other types of non-enzymatic proteins. From a molecular perspective, such fusion proteins are challenging targets for inhibitor development. Traditional drugs belong either to the group of biologics including antibodies, peptides, nucleic acids or vaccines or to the group of small molecules [14]. While the former are only suitable to target cell surface or secreted proteins, the latter are designed to bind into deep hydrophobic pockets of proteins, such as the enzymatic cleft of enzymes. Hence, both classes of drugs are less suitable for TFs, as well as many other proteins, which are intracellularly located and mediate their physiological function via protein-DNA and protein–protein interaction. Interaction surfaces are normally large and unaffected by conventional small molecule inhibitors. Furthermore, with the exception of the DNA binding domains, large parts of TFs are

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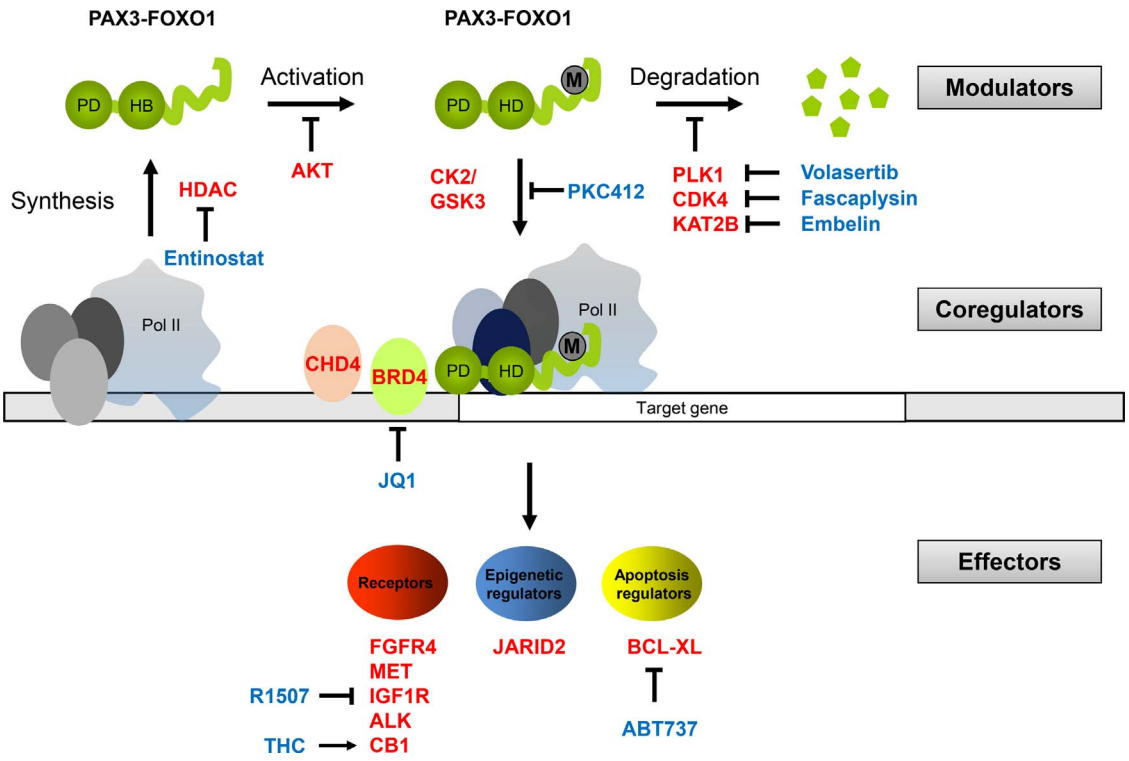

Fig. 1. Scheme depicting the different molecular levels potentially amenable for indirect targeting of fusion TFs using the example of PAX3-FOXO1. Proteins depicted in red are relevant for PAX3-FOXO1 activity in aRMS and are discussed in the text. Inhibitors and agonists discussed in the text are depicted in blue. PD, paired domain; HD, homeodomain; M, modification.

often intrinsically disordered and hence their 3D structure is labile and dependent on interactions with functional partners [15]. Based on this low druggability, direct inhibition of TFs was claimed to be very difficult. This fact is also exemplified by the decades-long, but so far unfruitful efforts to identify drugs that would directly inhibit the oncogene c-MYC [16]. Furthermore, experimental approaches including mRNA targeting using nucleic acid based therapeutics (antisense oligonucleotide or siRNA) that work well in *in vitro* model systems [17,18], might not be easily translatable to the clinics. However, activation or repression of target gene transcription does not only depend on the fusion TF itself, rather these proteins act in concert with many other proteins, including epigenetic regulators as well as the basic transcriptional machinery. At this level, one might be able to identify druggable targets amenable for indirect interference with TF activity. In addition, also synthesis and degradation pathways, as well as potential activation steps by post-translational modifications and/or relevant, selected downstream targets might represent potential levels for inhibition (Fig. 1). Hence, we argue that a deeper understanding of the biology of the individual fusion proteins might lead to identification of novel therapeutic targets.

2. FOXO translocations in alveolar rhabdomyosarcoma

Several members of the FOXO family are involved in translocations leading to generation of oncogenic fusion TFs. These include MLL-FOXO3/4 found in leukemias and PAX-FOXO1/4 fusions found both in sinonasal sarcoma and alveolar rhabdomyosarcoma (aRMS) [19–24]. In all cases the C-terminal transactivation domain of the FOXO protein is fused to the N-terminal DNA binding domain of the fusion partner. The resulting fusion protein acts as highly active TF inducing aberrant expression of target genes of the DNA-binding partner protein. One of the best studied among these fusion proteins is PAX3-FOXO1 in aRMS and therefore development of therapies based on functional knowledge of its biology might serve as blueprint for other fusion TFs.

aRMS belongs to the group of rhabdomyosarcoma (RMS) which are the most common soft tissue sarcoma in children accounting for 3% of all childhood and 2% of all adolescent cancers [25]. According to the latest WHO classification, RMS includes the subtypes alveolar (aRMS), embryonal (eRMS), spindle cell/sclerosing (sRMS/scRMS) and pleomorphic RMS (pRMS). While all these tumors are characterized by myogenic features, the different subgroups are clearly distinct by histological, molecular and clinicopathological characteristics.

Current therapy of RMS is still based on conventional strategies including chemotherapy, surgery and radiotherapy. While in some subgroups such as eRMS, application of these therapies increased the 5-year overall survival to more than 70%, efficacy in other subgroups is still much lower and 5-year overall survival remains dismal (< 50%) [26]. Especially in case of aRMS, novel therapeutics are urgently needed.

60% of aRMS cases bear PAX3-FOXO1 fusion proteins and a homologous fusion PAX7-FOXO1 is detected in another 20%, while alternative fusions of PAX3 with FOXO4, NCOA1/2, Gli2, INO80 have been found in a smaller number of cases [2,24,27–29]. The corresponding wildtype proteins have been intensely studied in the past. The PAX family TFs (PAX1-9) act as regulators of lineage commitment and tissue development during embryogenesis [30]. PAX3 is involved in regulation of neural tube, neural crest and skeletal muscle development.

The main physiological role of the FOXO family of TFs (FOXO1, FOXO3, FOXO4 and FOXO6) is the induction of an adequate response to environmental changes to maintain homeostasis [31]. Under homeostatic conditions, FOXO TFs are transcriptionally inactivated by sequestration into the cytoplasm. A range of posttranslational modifications (phosphorylation, acetylation) are involved in induction of nuclear export. Different types of environmental stresses lead to nuclear translocation of FOXO proteins. This major activation step then initiates the transcription of target genes, which affect different cellular processes including cell cycle, cell survival and metabolism. In sum, this helps the cell to adapt to the new conditions [31].

The PAX3-FOXO1 fusion protein is composed of two N-terminal

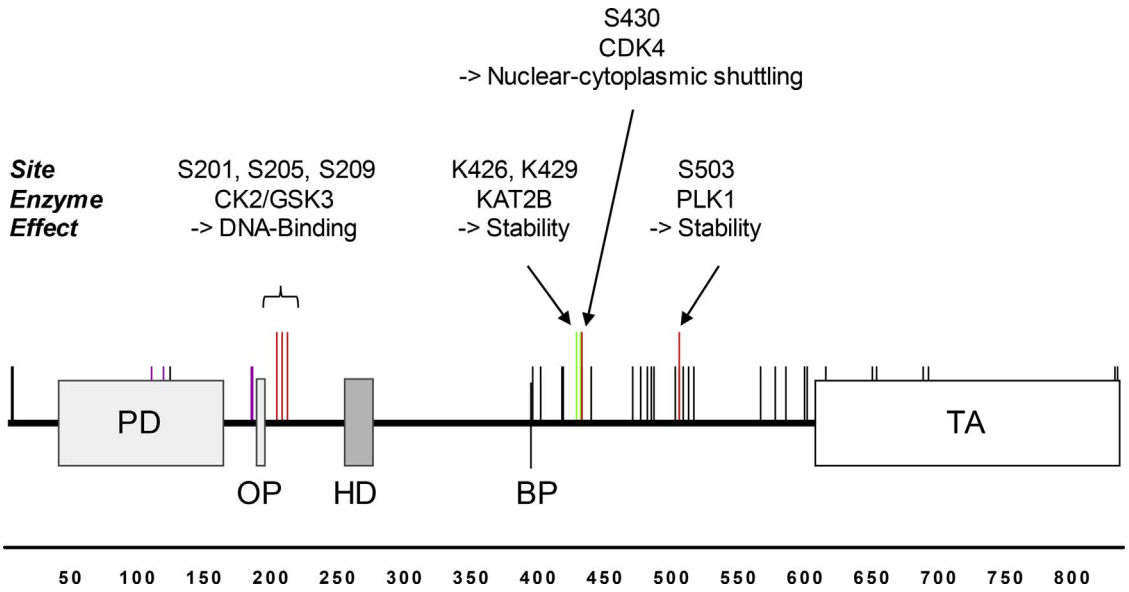

Fig. 2. Scheme depicting posttranslational modifications and their potential influence on PAX3-FOXO1 function. The locations of the individual modified residues on the PAX3-FOXO1 sequence are indicated by vertical lines. Red and green lines indicate phosphorylation and acetylation sites detected in PAX3-FOXO1, respectively. Residues discussed in the text are labeled above the illustration, together with involved enzymes and their role in PAX3-FOXO1 function. Black and purple lines indicate phosphorylation and acetylation sites detected in PAX3 or FOXO1, respectively, but not (yet) in PAX3-FOXO1. PD, paired domain; OP, octapeptide; HB, homeodomain; BP, breakpoint; TA, transactivation domain.

DNA binding domains of PAX3 (paired box domain and paired-type homeodomain) and the C-terminal transactivation domain of FOXO1. Via the PAX3 domains, the protein binds to more than one thousand sites in the genome, mostly promoter-distal enhancer regions enriched for PAX3 motifs [32] thereby causing transcriptional activation of several hundred target genes. The target gene signature contains different myogenic as well as neuronal markers, reflecting the physiological function of the wildtype PAX3 protein [27,33,34]. This also supports the hypothesis that the cell of origin of aRMS is a cell of the myogenic lineage and that the fusion protein blocks terminal differentiation. Among the targets are also different potentially druggable proteins such as several receptor tyrosine kinases (FGFR2, FGFR4, IGF1R, ALK, MET) and G-protein coupled receptors (CB1, ADRA2C) (for a comprehensive review on PAX3-FOXO1 target genes see [35]).

Apart from the fusion proteins, the mutational landscape of aRMS tumors is very quiet with an average of only 6.4 somatic mutations per tumor, from which only 2.5 mutations (median) are in transcribed genes. Again only a fraction of them are cancer-associated and druggable [2]. Importantly however, genomic analyses mainly focused on the coding part of the genome. In-depth analysis of the non-coding part of the genome will be necessary to test for somatic mutations in regulatory regions that are involved in RMS tumorigenesis. Furthermore, the involvement of inherited genetic changes needs more attention in the future. Druggable proteins boosted by such genetic changes might represent another class of potentially suitable targets. In the meantime, druggable driver genes are available in only a small number of cases for potential therapy. These include mutant PIK3CA (in about 2% of cases) or amplified CDK4 (amplified in about 12% of cases) [2,36,37]. However, these mutations represent secondary hits that appear after generation of the translocation and therefore might be present only in subclones of the tumor [3]. Furthermore, functional validation of CDK4 as therapeutic target in RMS revealed an inverse correlation between CDK4 expression levels and sensitivity towards CDK4 inhibitors, suggesting that therapeutic decisions cannot be based solely on genetic information, but careful functional characterization of cellular dependencies is an important prerequisite [38].

In contrast, different studies have shown that human aRMS tumor cells depend on continuous activity of the fusion protein [4,33,39]. Therefore, both from a genetic and functional point of view the PAX-FOXO1 fusion proteins are the most promising targets in the large majority of aRMS cases.

Along these lines, we summarize here the current knowledge regarding the different layers of regulatory mechanisms of the PAX3-FOXO1 fusion protein including modulators, co-regulators, and effectors and discuss their potential for therapeutic interventions (Fig. 1).

3. Mechanisms regulating PAX3-FOXO1 activity and their potential for therapeutic intervention

3.1. Modulators

In general, modulators of fusion TFs include proteins involved in synthesis and degradation pathways as well as proteins involved in potential activation/inhibition mechanisms (Fig. 1). We will discuss here these different aspects of the fusion protein biology and compare the mechanisms to the wild type proteins, if information is available.

Currently, not much is known about mechanisms controlling the transcription of the fusion protein. While upstream enhancers that drive PAX3 expression in the myogenic or neuronal lineage have been identified [40,41], their involvement in transcriptional control of the fusion protein in aRMS cells remains to be investigated. However, it has been reported that HDAC inhibition by Entinostat leads to decreased transcription of the fusion protein and slows tumor growth in xenograft experiments [42]. In addition to Entinostat, which has not entered the clinical stage yet, also the clinically approved HDAC inhibitors Panobinostat and Vorinostat have been shown to affect aRMS cell viability [43]. However, since HDAC inhibitors might induce a complex combination of downstream effects, more research is needed to clarify the order and interplay of events leading to reduction of PAX3-FOXO1 transcription and cell viability under these conditions.

More information is available about regulatory mechanisms exerting their function at the post-translational level. A plethora of modification sites has been identified in wild type FOXO1 [44]. Many of them are located in its C-terminal half and are therefore also present in the fusion protein, including at least 29 phosphorylation, 8 acetylation, 2 methylation and 5 O-GlcNAc sites (Fig. 2) [44]. This indicates that a complex enzymatic network is regulating FOXO1 activity. While many of the modifications have only been detected by high throughput mass spectrometry or after *in vitro* enzymatic assays [44] and are not yet functionally validated, for several of these sites involvement in

subcellular localization and degradation of FOXO1 could be shown. Interestingly, among these are conserved AKT phosphorylation sites (S437/S500 in PAX3-FOXO1) as well as sites phosphorylated by additional kinases that all regulate nuclear-export and proteasomal degradation of FOXO1 (for a comprehensive review on this topic see [45]). Importantly however, in context of the fusion protein the effect of these modifications on subcellular localization are overridden by a strong nuclear localization signal present in the PAX3 domain [46] and their potential physiological role remains to be demonstrated.

Indeed, regulation of degradation seems to be one major aspect that differs considerably between PAX3-FOXO1 and wildtype PAX3 and FOXO1 proteins and this might contribute to the oncogenic effect of the fusion protein. First evidence for this came from differentiating as well as proliferating myoblast cells where (exogenous) PAX3-FOXO1 was shown to be more stable than both endo- and exogenous PAX3. The difference has been assigned to a monoubiquitination site relevant for proteasomal degradation, which is present in the C-terminal half of PAX3 and therefore absent in the fusion protein [47,48]. However, also PAX3-FOXO1 protein levels were subsequently shown to be under control of the Ubiquitin-proteasome system since PAX3-FOXO1 levels can be stabilized by proteasome inhibitors such as MG-132 in aRMS cells [49–51]. Furthermore, endogenous PAX3-FOXO1 is able to destabilize the TF EGR1 by a mechanism involving direct interaction and subsequent co-degradation via the proteasome, from which it was concluded that the fusion protein is relatively unstable in aRMS cells [50,51]. Along these lines, several post-translational modifications have been identified that might influence fusion protein stability. First, phosphorylation by AKT at unknown sites was shown to inhibit the activity of the fusion protein [52], and AKT activation via Thapsigargin-treatment led to reduced DNA binding activity and subsequently to degradation of the fusion protein in aRMS cells [53]. Second, in a combined screening approach with both a kinome-wide siRNA library and a large drug library containing mostly kinase inhibitors, PLK1 was identified as a regulator of PAX3-FOXO1 protein stability [49]. Phosphorylation of Ser503 by PLK1 stabilizes the PAX3-FOXO1 protein and its inhibition leads to rapid degradation of the fusion protein via the proteasomal pathway. PLK1 expression is regulated in a cell cycle dependent manner and increases from late S to M phase and its activity peaks during mitosis. Since also transcription of PAX3-FOXO1 has been shown to peak in G2 [54], PAX3-FOXO1 levels seem to be upregulated during G2/M phase of the cell cycle by mechanisms acting both at the mRNA and protein level. This upregulation has been linked with checkpoint adaption, allowing the cell to transit the G2/M checkpoint despite presence of unrepaired cellular damage [54], an effect of PAX3-FOXO1 with potential clinical implications taking into account that DNA-damage induced cell cycle arrest and apoptosis is a central aim of conventional chemo- and radiotherapy [54].

Interestingly, besides PLK1, different other enzymes have been shown to modify PAX3-FOXO1 in the same region. Among them is the acetyltransferase KAT2B (P/CAF) which, via acetylation of two nearby lysine residues (K426/K429), has also been shown to have a stabilization effect on PAX3-FOXO1 [55]. In wildtype FOXO1, acetylation of these lysines stimulates phosphorylation of the nearby Ser256 (Ser437 in PAX3-FOXO1) [56] and subsequent nuclear export as well as Skp2-mediated ubiquitination and degradation [57], again pointing to differences between wild type FOXO1 and the fusion protein. Importantly, genetic and pharmacological interference with both PLK1 (inhibitor Volasertib) and P/CAF (inhibitor Embelin) reduced aRMS cell viability *in vitro* and xenograft growth in mice, in case of Volasertib treatment manifesting even as dramatic tumor regression [49,55].

Finally, CDK4 has been claimed to phosphorylate Ser430 in PAX3-FOXO1 and its inhibition with Fascaplysin led to redistribution of the fusion protein to the cytoplasm, thereby reducing its transcriptional activity [58]. Whether the clinically approved CDK4/6 inhibitor Palbociclib, which has been shown to inhibit aRMS cell proliferation *in vitro* and to delay xenograft growth in mice [59], has similar effects, needs further clarification. Taken together, these data suggest that modifications of the breakpoint-proximal region of the FOXO1 domain might affect localization and/or stability of PAX3-FOXO1 (Fig. 2). Therefore, pharmacological induction of degradation might be a potential approach for aRMS therapy. Further characterization of the molecular mechanisms regulating PAX3-FOXO1 stability such as identification of ubiquinated lysine residue(s) and components of the degradation machinery (E3 ligases and de-ubiquinating enzymes) might reveal additional important possibilities to pharmacologically enhance degradation of the fusion protein.

Apart from protein stability, different groups also described some activation mechanisms of PAX3-FOXO1. One mechanism seems to involve several phosphorylation events that take place in the PAX3 domain of the fusion protein (Fig. 2) [44]. Modified sites are located in the conserved octapeptide region located between the two DNA binding domains of both PAX3 and PAX3-FOXO1 [60–62]. The FDA-approved kinase inhibitor PKC412 (Midostaurin) interferes with phosphorylation in this region, an effect which is accompanied by both efficient induction of cell death and inhibition of PAX3-FOXO1 activity, at least at some target genes [60]. While others have linked this region with protein–protein interaction [63], it was shown that inhibition of phosphorylation reduces DNA binding and consequently transactivation activity of the fusion protein [60]. Since PKC412, as derivative of Staurosporine, affects a broad spectrum of kinases, the identity of the involved kinase(s) is not clear yet. However, the phosphorylated peptide contains six potential phosphorylation sites, which are present in the very regular order Ser187xxxxxSer193xxxSer197xxxSer201xxx-Ser205xxxSer209. The motif SerxxxSer(P) represents a consensus phosphorylation motif for the kinase GSK3 [64]. For efficient phosphorylation by GSK3, the C-terminal Ser is normally phosphorylated by a priming kinase. In some cases, such as the very similar 5-Ser motif present in muscle glycogen synthase, this priming step is done by CK2 [65,66]. In agreement with such cooperation, phosphorylation of wildtype PAX3 at Ser201 and Ser205 in myoblasts was found to depend on GSK3 and CK2, respectively [61,62]. Furthermore, two recent publications showed that different GSK3 inhibitors were able to reduce phosphorylation of Ser201 in endogenous PAX3-FOXO1 and also to affect viability of aRMS cells [67,68]. This suggests that GSK3 indeed could be (one of) the kinase(s) phosphorylating this peptide. Since PKC412 as well as GSK3-inhibitors potentially interfere with cellular physiology also at other levels, it is not clear yet to which extent reduction of PAX3-FOXO1 activity contributes to the induction of cell death. Hence, further validation of these results is necessary before they can potentially be applied as aRMS therapy. However, these first findings identifying regulatory mechanisms affecting PAX3 activity via modulation of DNA binding may open the door for more specific ways to interfere with fusion protein activity.

3.2. Co-regulators

While numerous interactors/co-regulators of the wildtype contributors to the PAX3-FOXO1 fusion, particularly of FOXO1, have already been described [69], identification of co-regulators of PAX3-FOXO1 in aRMS cells was a rather neglected area of research. Only recently, insights from few novel studies are now beginning to shed some light on this.

In one of these studies, CHD4 has been identified as co-regulator of PAX3-FOXO1 for a subset of its target genes and CHD4 silencing was shown to efficiently induce aRMS cell death [70]. CHD4 is a multidomain protein containing two plant homeodomains (PHD), two chromodomains and a SNF2-like ATPase domain, the latter defining this protein as a member of the ATP-dependent chromatin remodeling protein family. Chromatin remodelers use the energy of ATP hydrolysis to move, destabilize, eject, or restructure the nucleosomes in the chromatin. Thereby they affect packaging/accessibility of the DNA for the molecular machineries involved in replication, repair as well as

transcription. CHD4, like its homologues CHD3 and CHD5, is a peripheral component of the Nucleosome Remodeling Deacetylase (NuRD) complex [71]. Initially, this large complex was mainly associated with transcriptional repression, but it is now appreciated that its effects on gene expression are more complex and can also include stimulation of gene expression [72]. Interestingly, CHD4 was associated in different studies with an activating function of NuRD. In the neuronal lineage it was shown that the identity of the CHD protein present in the NuRD complex determines its influence on gene expression and cellular behavior [73]. In progenitors, CHD4 is a component of the NuRD complex, induces expression of key regulators of neuronal development such as PAX6 and SOX2 and promotes cell proliferation. The switch to CHD5 and CHD3 at later developmental stages induces repression of the very same genes and is associated with migration and differentiation of the cells, respectively [73]. In agreement with this model is data from mouse embryonic stem cells where CHD4 has been found to bind to the −1 and +1 nucleosomes flanking nuclear free regions (NFR) around the transcriptional start site of a subset of active genes [74]. Importantly, depletion of CHD4 resulted in reduction of RNA Polymerase II (Pol II) levels at these sites, suggesting that presence of CHD4 is of general importance for recruitment of the transcriptional machinery [74]. The mechanisms by which CHD4 co-activates transcription of PAX3-FOXO1 target genes in aRMS would also support these characteristics [70] since CHD4 does not directly interact with PAX3-FOXO1 but binds to sites in the chromatin located in close distance to PAX3-FOXO1 occupied sites. Based on ChIP qPCR studies it was postulated that binding of CHD4 is necessary for subsequent landing of PAX3-FOXO1, potentially allowing PAX3-FOXO1 to activate transcription. A handful of other studies demonstrated similar collaborative interplays of CHD4 with TFs such as GATA3 in T-helper 2 cells or CLOCK-BMAL1 in mouse tissue [75,76]. On the contrary, CHD4 has also been associated with repression of gene expression. In colorectal cancer cells, it is involved in a DNA-damage response mechanism leading to general transcriptional repression [77]. CHD4 is recruited via OGG1 and ZMYND8 to sites of oxidative DNA damage and double strand breaks, respectively. It co-recruits transcriptional repressor enzymes including the DNA methyltransferases DNMT1, DNMT3A, and DNMT3 B as well as the histone methyltransferases EZH2 and G9a. By depositing repressive chromatin modifications e.g. in promoters of tumor suppressor genes, this CHD4 complex promotes tumorigenesis [77,78]. The relevance of such repressive activity of CHD4 in aRMS cells, however, is not clear yet. Nevertheless, taken together, these data highlight CHD4 as potential therapeutic target in aRMS affecting PAX3-FOXO1 activity.

In another recent study, BRD4 was identified as co-regulator of PAX3-FOXO1 [79]. BRD4 is a versatile activator of transcription at the level of the chromatin. The protein has histone acetyltransferase (HAT) activity involved in nucleosomal eviction and chromatin decompaction at gene promoters as well as an atypical kinase activity phosphorylating the C-terminal domain of Pol II, thereby regulating promoter pause-release and transcriptional elongation [80,81]. The small molecule inhibitor JQ1 interferes with chromatin binding of BRD4 by blocking its bromodomain, which normally recruits this protein to active genomic sites with high histone acetylation levels. Although BRD4 occupies a large part of all active regulatory elements in the genome, treatment with JQ1 leads to preferential loss of BRD4 from the largest and most active enhancers ("super-enhancers (SE)")[82]. Since SE drive the expression of key oncogenes in many tumors, tumor cells often show an enhanced sensitivity towards JQ1 [82,83]. In accordance with these concepts, it was found in aRMS cells that BRD4 and PAX3-FOXO1 collaborate particularly at SE-driven target genes of the fusion protein. Hence, JQ1 and other BRD4 inhibitors suppress a large part of the target gene signature of PAX3-FOXO1 and induce death of aRMS cells *in vitro* and tumor growth suppression *in vivo*. Mechanistically, BRD4 was shown to interact with PAX3-FOXO1 and its inhibition by JQ1 leads to depletion of both BRD4 and PAX3-FOXO1 from co-occupied genomic sites as well as a dramatic reduction of the half-life of PAX3-FOXO1. Apart from interference with the activity of PAX3-FOXO1 and potentially other TFs, JQ1 has also been associated with inhibition of angiogenesis via direct effects on endothelial cells in aRMS xenograft tumors [84], suggesting that its tumor suppressive effect is the result of diverse mechanisms. Interestingly however, both CHD4 and BRD4 have no influence on transcription of PAX3-FOXO1 itself, pointing at differences in regulation of the fusion protein and its major downstream targets.

From a conceptual point of view the outcome of blocking CHD4 or BRD4 in aRMS cells seem to be very similar. For both their influence on the transcriptome goes beyond fusion protein target genes. Ultimately, inhibition might lead to induction of transcriptional chaos, which drives cancer cells into a death program. Such effects could be of general significance for transcription-addicted cancers [85]. Indeed, available BRD4 inhibitors might be of therapeutic value in a range of tumors, often associated with downregulation of driver oncogenes [82,83]. Hence, BRD4 targeting is an already quite advanced approach and first clinical trials are ongoing. Also CHD4 blockade has been found to affect viability of a range of cancer types including breast and liver cancer or AML [86–88]. However, in contrast to BRD4, no small molecule inhibitor for CHD4 is currently available. Importantly, some of the CHD4 domains are potentially druggable including the PHD- and the chromo-domains which mediate binding of the protein to unmodified or H3K9-methylated histones and DNA, respectively [89] as well as the helicase domain.

The influence of BRD4 and CHD4 as epigenetic regulators on PAX3-FOXO1 activity and aRMS cell viability raise the important question whether other epigenetic regulators have similar roles. Since many of them are druggable, such proteins could be interesting targets as well and more research in this area therefore holds a lot of promise.

3.3. Effectors

The level of target genes is the most extensively studied aspect of PAX3-FOXO1 biology during the last decade. A selection of PAX3-FOXO1 target genes with inhibitors available was tested as targets for therapy.

One type of target genes studied in this context are different cell surface receptors. Prime candidates are receptor tyrosine kinases (RTKs) such as IGF1R, FGFR4, ALK and MET, for which small molecule and/or antibody inhibitors are available. Inhibition of some of these kinases indeed revealed promising effects in preclinical models based on cell lines [90–95]. However, experience with the IGF1R inhibitory antibody R1507 in a phase II clinical study with Ewing and other sarcoma dampened the enthusiasm for application of these inhibitors [96], since the initial objective response rate of 10% was rather modest. While careful selection of patients based on biomarkers for treatment response might allow to increase this number, responses seen in sensitive cases were also only transient. Intrinsic and acquired resistances therefore play a major role and affect treatment outcome. Some of the resistance mechanisms involved were characterized later in *in vitro* studies and involved stabilization of IGF1R, heterodimerization of IGF1R with other receptor tyrosine kinases as well as activation of parallel or downstream kinase signaling pathways [97–100]. Hence, these data are in perfect agreement with the comprehensive experience gained with such drugs during the last decade when used for the treatment of carcinoma. There, it was learned that therapy responses are mainly seen in cases where the target is activated by mutation or gene amplification and acts as a driver in the corresponding tumor, such as in cases of BRAF V600E mutant melanoma or ERBB2/HER2-amplified tumors [101]. However, even then outgrow of resistant relapse tumors follows initial regression in virtually all cases. In aRMS however, despite being highly overexpressed as targets of the fusion protein, all the kinases discussed are usually wildtype and, with exception of ALK in some rare cases [102], not genetically amplified. This further limits their potential as therapeutic targets. Most likely, such

drugs should be used in combination with standard-of-care chemotherapy or other targeted drugs.

Another type of receptor that was investigated in a similar way was the cannabinoid receptor 1 (CB1) [103]. This was based on the finding that different tumors expressing high levels of CB1 including glioma, breast cancer and melanoma are sensitive towards CB1-agonists [104–106]. There, treatment with the natural cannabinoid Δ9-tetrahydrocannabinol (THC) or synthetic CB1-agonists lead to induction of cell death. In many of these cancers, the mechanism behind this effect involves induction of *de novo* synthesis of the sphingolipid ceramide, which via induction of ER stress first stimulates autophagy and subsequently apoptotic cell death (for review see [107]). Indeed, the same phenomenon was also detected in aRMS cells *in vitro* and *in vivo* [103]. Furthermore, CB1 inhibition also reduced lung metastasis formation in a PAX3-FOXO1-overexpression myoblast model [108]. Similar to RTKs however, activation of signaling pathways via overexpression of ALK or activation of ERK has been found to lead to resistance towards CB1 agonists in several types of tumors, hence this treatment might be associated with the same downsides as discussed above for RTKs [109,110].

A second type of target genes potentially suitable for therapeutic purposes are regulators of the apoptotic machinery. An interesting PAX3-FOXO1 target in this context is Bcl-XL [111]. This anti-apoptotic protein of the Bcl-2 family is upregulated in many tumors and blocks intrinsic apoptosis upstream of the mitochondria by neutralizing pro-apoptotic Bcl-2 family members. Bcl-XL inhibition therefore shifts the balance between these proteins towards induction of apoptosis. Importantly, PAX3-FOXO1 was shown to also upregulate the pro-apoptotic Bcl-2 family member NOXA in aRMS cells [112]. Potentially, this sets the cells into a state referred to as “primed” for apoptosis, which makes them especially amenable for apoptosis-directed drugs [113]. In agreement with this hypothesis is data showing that the cytotoxic effects of a series of drugs in aRMS cells is mediated (at least partially) by NOXA [112,114–117]. Different inhibitors are available for Bcl-XL targeting, with variable effects on other anti-apoptotic Bcl-2 family proteins. Their action might be especially useful in combination with other drugs to enhance induction of apoptosis. Indeed, ABT737, an inhibitor of Bcl-XL, Bcl-2 and Bcl-w, synergized with different inhibitors of the PI3 K/AKT/mTOR pathway in induction of apoptosis of aRMS cells *in vitro* [118]. The clinical development of Bcl-XL inhibitors however has been slowed down due to on-target induced thrombocytopenia [119,120], a side effect that might be circumvented by adjusting dosing schedules [121].

Along the lines described above, a third class of interesting target genes are epigenetic regulators, among them JARID2 [122]. JARID2 belongs to the Jumonji family of histone demethylases, however it lacks some critical active site residues and therefore doesn‘t have demethylase activity. Instead, it is thought to act as scaffold protein and to recruit repressive epigenetic complexes like PRC2 or other methyltransferase complexes including G9a/GLP and SETDB1 to the chromatin. Thereby it induces an increase of the repressive chromatin marks H3K27me3 and H3K9me3 [123–125]. This then leads to transcriptional silencing of the targeted genes. The core PRC2 complex is composed of the three proteins SUZ12, EED and the methyltransferase EZH2 or its close homologue EZH1. By orchestrating gene expression changes, PRC2 is a central regulator of cellular differentiation including the one along the myogenic lineage [126]. For proper muscle differentiation, a complex interplay between PRC2-EZH1 and PRC2-EZH2 at muscle differentiation genes takes place. The PRC2-EZH2 complex is removed while the PRC2-EZH1 complex is recruited to some muscle differentiation factors such as MYOG, and both these steps are required for activation of the early myogenic program via MYOD [127]. Most PRC2 components including EED, SUZ12 and EZH2, but not EZH1, are overexpressed in aRMS when compared to skeletal muscle cells [122,128]. Hence, it has been suggested that PRC2-EZH2 contributes to the undifferentiated phenotype of aRMS by repressing differentiation genes [122]. In accordance with this, it was shown that silencing of JARID2 in aRMS cells leads to reduced levels of H3K27me3 at myogenic differentiation genes including MYOG and MYL1 and induction of expression of these genes. Similar effects were seen after silencing of EED [122]. On the cellular level, JARID2 silencing leads to reduction of proliferation and induction of a differentiation phenotype of aRMS cells. Taken together, these data suggest that JARID2 mediates some of the well-known anti-differentiation effects of PAX3-FOXO1 in aRMS cells, potentially via PRC2. Since differentiation therapy has been found to be a successful approach in other tumors, most prominently in acute promyelocytic leukemia [129], these data might stimulate further evaluation of this form of therapy for aRMS. EZH2 is a druggable PRC2 component and different EZH2 inhibitors are currently under clinical development. Interestingly however, recent testing of the EZH2 inhibitor Tazemetostat by the pediatric preclinical testing program revealed no relevant effect on aRMS xenograft growth in mice [130]. Hence, deeper insights into epigenetic regulation of transcription are required to see whether and which PRC2-directed drugs would be suitable for aRMS differentiation therapy.

An important aspect that must be taken into account when interpreting the potential of downstream effectors of PAX3-FOXO1 as therapeutic targets is the fact that a physiological ranking of the different PAX3-FOXO1 target genes based on their relevance for tumorigenesis is still missing at this point. Since only a small fraction of target genes could be investigated so far and since the targets with highest relevance might also be most effective as targets for treatment, the most important findings in this area could still lie ahead.

4. Conclusions and perspectives

As outlined for PAX3-FOXO1 above, the biology of individual fusion TFs is complex and a lot of effort is needed to identify optimal strategies to interfere with their activity. Furthermore, the large variety of fusion TFs expressed by different tumor entities or sometimes even in one specific tumor type as well as rarity of many of these tumors complicate functional studies and slow down the therapeutic progress. However, different molecular techniques developed during the past few years may allow to address some important aspects of fusion TF biology in a more efficient way in the near future, therefore accelerating research. A prominent example is CRISPR-Cas9 mediated genome editing, which has developed into a plethora of downstream applications. Of special interest for fusion TF research could be knock-ins of reporters into target gene loci that can be used in genetic or pharmacologic screens to evaluate fusion protein activity. Other CRISPR-Cas9 applications include library screens for identification of the most relevant target genes or generation of chromosomal translocations in potential cells of origin. Further, CRISPR-Cas9 will also facilitate the generation of sophisticated genetically engineered mouse models, which will allow mimicking the human disease in more detail. Furthermore, connectivity maps might help to find drugs that affect the PAX3-FOXO1 induced gene expression program [131].

Also in the area of protein targeting recent developments might open novel possibilities. One approach is targeting of proteins by drugs that covalently bind to their target and do not rely on hydrophobic binding pockets [15]. Another potentially useful method is the proteolysis targeting chimera (PROTAC) technique used to induce specific degradation of proteins. Basis for this technology are chimaeric small molecules that combine a moiety that binds to the protein of interest with a second one that binds to an E3 ligase, thereby inducing ubiquitination and degradation of the protein of interest. The PROTAC technique was already described in 2001 [132], but early approaches lacked potency [133]. Recently however, it was re-awakened as promising approach for cancer treatment. This was based on the finding that a specific class of small molecules called Phtalimides, such as the infamous teratogenic compound Thalidomide, bind to the E3 ligase Cereblon and thereby establish a novel interface that leads to

recruitment and degradation of the transcription factors IKZF1/3 as well as CK1α. This effect is of therapeutic value in multiple myeloma and myelodysplastic syndrome, respectively [134–137]. By coupling Phtalimides with a second small molecule which specifically binds to a protein of interest, Cereblon–mediated degradation of any target protein in Cereblon-positive cells might be induced [138]. Another promising E3 ligase that can be hijacked in a similar way for specific degradation purposes is VHL [139,140]. Collectively, these techniques have the potential to shift the paradigms of the (un)druggability of fusion TFs and other proteins that are problematic to drug.

A potential alternative to genetically driven selection of therapies is represented by the functional screening for unrecognized vulnerabilities by direct drug-profiling of patient cancer cells. The most advanced protocols for such an approach involve transplantation of tumor material from individual patients as patient derived xenograft (PDX) into mice for amplification of the tumor material. Secondary transplants with the amplified tumor material can then be used for tests of (small numbers) of drugs *in vivo*. For high-throughput screening with larger drug libraries *in vitro* cultures of primary cells from PDX might be established. Since not all tumors engraft in mice and since each engraftment round normally takes one to several months of time, the optimal solution would be the establishment of primary cell cultures, either in 2D or 3D, directly from the patient material in a co-clinical approach. This approach however necessitates on the one hand sufficient amounts of untreated tumor material and on the other hand culture conditions preventing overgrowth of stromal cells such as fibroblasts.

Despite all these promising novel techniques for target/drug identification, a major therapeutic hurdle is the development of drug resistance. Especially when applied as single agent, development of resistance against targeted drugs is very often inevitable. Numerous different mechanisms have been described to be involved in therapy resistance, including changes in drug metabolism or circumventing dependency on the drug target (summarized in [141]). Concerning the latter, loss of dependency on PAX3-FOXO1 was recently described in a study using a human myoblast based aRMS model [142]. There, to mimic aRMS development, myoblasts were transduced with doxycycline-inducible PAX3-FOXO1 and MYCN. Cells expressing the two oncogenes were found to efficiently form xenograft tumors in mice. Furthermore, silencing of PAX3-FOXO1 expression upon doxycycline-withdrawal as expected induced tumor regression. Unexpectedly however, a minor population of cells in some of the established tumors was found to survive PAX3-FOXO1 depletion and generate PAX3-FOXO1 negative relapse tumors [142]. Whether human aRMS tumors also contain cells that are not PAX3-FOXO1-dependent however needs further clarification. A common way to circumvent development of resistance to therapy makes use of drug combinations, an approach that was shown to be much less prone to develop therapy resistances [143]. PAX3-FOXO1 directed drugs in combination with standard-of-care therapy might be an option to eradicate resistant cells upfront. In the long run, the knowledge of different molecular angles to interfere with PAX3-FOXO1 activity however opens the possibility for combinations of drugs that act at different molecular levels.

Overall, the data reviewed here highlights that a period of genetic analyses of the cancer genome, performed during the past two decades, has facilitated the identification of the drivers of tumorigenesis in numerous tumors. Now a period of intense functional studies is necessary to find ways to interfere with these proteins. In case of oncogenic fusion TFs, the exploration of the biological context could be one route to zoom in on these difficult to drug molecules and find angles for a therapeutic intervention. Another route might be opened by novel protein targeting techniques. Approaches combining knowledge-based drugs with functional drug profiling to identify unrecognized tumor specific vulnerabilities might represent the most successful approaches in the future.

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